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LOCAL INTERNEURONS DEFINE FUNCTIONALLY DISTINCT REGIONS WITHIN LOBSTER OLFACTORY GLOMERULI

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Summary

Whole-cell recording coupled with biocytin injection revealed four types of interneurons intrinsic to the olfactory lobe (OL) of the spiny lobster *Panulirus argus*. Each type of neuron had a distinct pattern of arborization within the three anatomically defined regions of OL glomeruli (cap, subcap and base). Type I interneurons innervated all three regions, while types II, III and IV branched only in the cap, subcap and base, respectively. Type I interneurons responded to electrical stimulation of the antennular (olfactory) nerve with a burst of 1–20 action potentials and a 1–10 s depolarization. Type II (cap) interneurons responded to the same input with a burst of 1–3 action potentials followed by a shorter hyperpolarization. Type III (subcap) interneurons responded with a burst of 1–6 action potentials followed by a delayed, 0.5–4 s depolarization. Type IV (base) interneurons responded with a brief depolarization or a burst of 1–3 action potentials followed by a 1 s hyperpolarization. The regionalized arborization and the different response properties of the type II, III and IV interneurons strongly imply that lobster olfactory glomeruli contain functionally distinct regions, a feature that should be useful in understanding the multiple synaptic pathways involved in processing olfactory input.

Key words: lobster, *Panulirus argus*, interneuron, olfactory glomerulus, branching pattern, olfaction.

Introduction

Glomeruli are characteristic anatomical features of the first synaptic level of the olfactory pathway in a phylogenetically broad range of animals, including molluscs, arthropods and vertebrates (Ache, 1991; Hildebrand, 1995). While the role of glomeruli in olfactory coding remains unclear, glomeruli are widely believed to represent functional units in processing olfactory information (Ernst and Boeckh, 1983; Shipley and Ennis, 1996). In mammals, receptor cells expressing the same olfactory receptor protein converge onto a single glomerulus (Ressler *et al.* 1994), as do insect receptor cells with the same sensitivity to pheromone components (Hansson *et al.* 1992). This convergence of the primary receptor cells onto glomeruli is thought to play an important role in shaping the responses of olfactory projection neurons, the output neurons of this level.

The responses of olfactory projection neurons to odors are further shaped by intrinsic, inter- and intraglomerular processing mediated by local interneurons (Wellis and Scott, 1990; Christensen *et al.* 1993; Duchamp-Viret and Duchamp, 1993; Yokoi *et al.* 1995). As the glomeruli are the sites of synaptic contact between receptor cell axons, projection neurons and several classes of local interneurons (Pinching and Powell, 1971; Ernst and Boeckh, 1983; Boeckh *et al.* 1990), such processing can be complex and involve multiple synaptic pathways. Correspondingly, perhaps, olfactory glomeruli in mammals are not homogeneous structures with respect to intrinsic processing, since receptor cell axon terminals, local interneurons and projection neuron (mitral cell) primary dendrites can have spatially restricted domains within a single glomerulus (Takami and Graziedei, 1991; Hálasz and Greer, 1993; Greer *et al.* 1995; Shipley and Ennis, 1996). However, earlier studies have failed to demonstrate functional heterogeneity within glomeruli (Lancet *et al.* 1982), possibly because of the small size and non-uniform distribution of glomerular subcompartments in mammals, and the relationship between glomerular compartmentalization and intrinsic olfactory processing remains unclear.

Glomeruli in many arthropods show clearer anatomical heterogeneity so that two distinct tissue layers are easily recognizable in the spherical glomeruli of the honeybee *Apis*

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mellifera (Gascuel and Masson, 1991), and three layers are present in the columnar glomeruli of decapod crustaceans (Blaustein *et al.* 1988). Local interneurons and projection neurons branch preferentially in one or more of these layers (Fonta *et al.* 1993; Sun *et al.* 1993; Schmidt and Ache, 1996; Wachowiak *et al.* 1996), suggesting that the different glomerular regions may encompass different steps in glomerular processing. However, the functional significance of this glomerular compartmentalization is not clear, since the local interneurons mediating intrinsic processing in arthropods remain poorly characterized both anatomically and physiologically.

The present study is a first step towards understanding the role of local interneurons in glomerular processing in the crustacean olfactory lobe (OL). The lobster OL consists of approximately 1100 radially oriented, columnar glomeruli, each approximately 100 μm in diameter (Fig. 1A). Histological staining reveals three distinct anatomical zones within each glomerulus – an outermost cap region, approximately 50 μm thick, an underlying subcap, approximately 30 μm thick, and a proximal base, approximately 170 μm thick (Blaustein *et al.* 1988; Schmidt and Ache, 1992) (Fig. 1B,C). Here, we characterize four classes of neurons intrinsic to the OL of the spiny lobster *Panulirus argus* according to their branching patterns within OL glomeruli and their responses to electrical stimulation of the olfactory (antennular) nerve. We show that three of the neuron types branch selectively in distinct regions of the glomerulus and respond differently to electrical stimulation of the olfactory nerve. These results strongly imply that individual OL glomeruli are functionally heterogeneous with respect to olfactory processing and that the different anatomical regions represent sites of distinct processing events. This organization may be especially useful in understanding the multiple synaptic pathways within olfactory glomeruli and their respective roles in processing olfactory input.

Materials and methods

Preparation

Adult specimens of the Caribbean spiny lobster *Panulirus argus* Latreille were collected in the Florida Keys and held in the laboratory in running sea water. Medium-sized (50–70 mm carapace length), intermolt lobsters were used for all experiments. A perfused, isolated brain preparation, described previously (Wachowiak and Ache, 1994), was used to obtain whole-cell recordings from the somata of olfactory lobe (OL) neurons while electrically stimulating the antennular (olfactory) nerve. This preparation allowed simultaneous morphological and physiological characterization of the neurons without disturbing synaptic connections within the OL. Briefly, the brain was cannulated and continuously perfused with oxygenated *P. argus* saline, then dissected out of the head capsule and pinned down in a recording chamber that was subsequently mounted on the stage of a compound microscope (Axioskop, Zeiss). Somata were visualized with conventional bright-field optics at 400×.

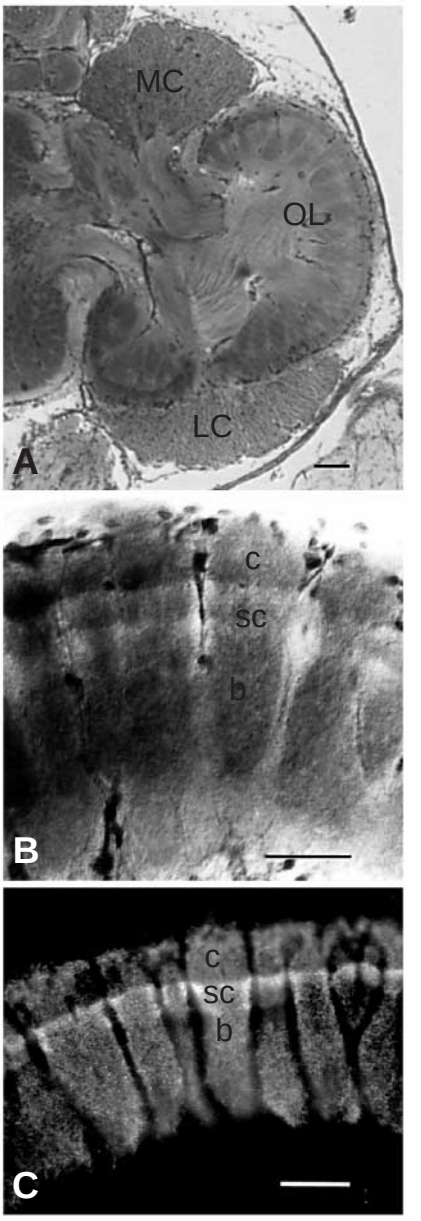

Fig. 1. Structure of the lobster olfactory lobe (OL) and glomeruli. (A) Thin (20 μm) section through the lobster OL, stained with ethyl gallate. Glomeruli are columnar in shape and oriented radially around the periphery of the OL. Local interneuron somata are located in the medial soma cluster (MC). LC, lateral soma cluster. (B) Higher-power view of several glomeruli showing the base (b), subcap (sc) and cap (c) regions. (C) Thick (100 μm) section through OL glomeruli, stained with an antibody to histamine and visualized with a CY-3-conjugated secondary antibody (see Materials and methods). Histamine-like immunoreactivity reveals boundaries between glomeruli and allows clear visualization of the base, subcap and cap. Ethyl-gallate-stained sections in A and B were kindly provided by Dr M. Schmidt. Scale bars, 100 μm.

Recording, stimulation and data acquisition

The somata of neurons intrinsic to the OL are contained in somata cluster 9 (Sandeman *et al.* 1992), herein termed the medial cluster for convenience. Gigaohm seals were formed on

medial cluster somata with borosilicate glass patch electrodes having tip diameters of approximately 1 μm (bubble no. 5; Mittman *et al.* 1987). Electrodes were coated with Sylgard to reduce capacitance and fire-polished immediately before use. In most cells, injection of less than 20 pA of hyperpolarizing current was required to maintain a membrane potential of –60 mV. Cells requiring current injection of more than 60 pA were used only for measurement of action potential latency and number. Responses to electrical stimulation of the antennular nerve were recorded in current-clamp mode, as described previously (Wachowiak and Ache, 1994). Signals were amplified with a patch amplifier (Dagan 8900), viewed on a storage oscilloscope and recorded digitally using pCLAMP software (Axon Instruments, Inc.) and an IBM-compatible computer. Results of experiments were also stored on video tape for further analysis at a high sampling rate using digital acquisition software (Axotape, Axon Instruments Inc.).

Intracellular staining

Individual neurons were stained by including 4 % biocytin (Molecular Probes, Eugene, OR, USA) in the patch solution, which passively filled the cell during recording. On termination of the recording, the perfused brain was allowed to sit undisturbed for 30–60 min, then fixed *in vitro* by replacing the saline bath with 4 % paraformaldehyde in 0.1 mol l^{-1} Sorensen phosphate buffer (SPB) + 15 % sucrose. After three paraformaldehyde rinses, perfusion was stopped and the brain was fixed overnight in paraformaldehyde. Further processing followed the procedure for biocytin staining detailed in Schmidt and Ache (1992). In some preparations, the biocytin staining was amplified using a biotin amplification procedure (Adams, 1992). After an initial incubation in ABC solution (Vector Labs, Burlingame, CA, USA), sections were reacted with H_2O_2 and biotinylated tyramine (3.75 mg ml^{-1}) for 8 min. After rinsing, the sections were reincubated in ABC and processed as normal. Most cells were counterstained with OsO_4 (4 %) to allow visualization of glomeruli. In a few cases, biotin staining was visualized using an avidin–Texas Red conjugate, and histamine-like immunoreactivity was visualized simultaneously using a CY-3-conjugated secondary antibody, as described in Wachowiak and Ache (1997). The unique pattern of histamine-like immunoreactivity (Fig. 1C), reported previously (Wachowiak and Ache, 1997), was useful in demonstrating the different patterns of glomerular innervation by the interneurons.

Strict attention was paid to the completeness of the fills, which was evaluated on the basis of two criteria: (1) termination of all branches within OL glomeruli and not within the core of the OL, and (2) the presence of branches which terminated while still darkly stained. Cells meeting both criteria were used in the initial classification and detailed characterization of interneuron types. The majority of filled cells met both of these criteria. Because of the clear differences between different morphological types (see Results), the few cells meeting only the first of these criteria could still be classified morphologically and used in the physiological characterization of the interneurons. Filled cells were reconstructed using a *camera lucida* attachment and photographed using bright-field or Nomarski optics. The number of glomeruli innervated by individual filled neurons was either counted directly or estimated by calculating the percentage of innervated glomeruli in each 100 μm section.

Solutions

Panulirus argus saline consisted of (in mmol l^{-1}): 460 NaCl, 13 KCl, 13 $CaCl_2$, 10 $MgCl_2$, 14 Na_2SO_4, 1.7 glucose, 3 Hepes, pH 7.4. Patch solution consisted of (in mmol l^{-1}): 30 NaCl, 11 EGTA, 10 Hepes, 3.7 $CaCl_2$, 180 potassium acetate, 696 glucose, brought to pH 7.0 with 5 mol l^{-1} KOH. Chemicals were obtained from Sigma.

Results

Forty-two neurons intrinsic to the OL were filled with biocytin and characterized according to their branching patterns within olfactory glomeruli. The neurons formed four distinct classes on the basis of their selective innervation of the base, subcap and cap regions of OL glomeruli. The branching patterns of the four classes were distinct enough that classification did not require a formal quantitative analysis of branch distribution. A subset of these neurons was also characterized according to their responses to electrical stimulation of the antennular nerve. Each morphological class had a unique response to electrical stimulation of the antennular nerve.

Neurons innervating the base, subcap and cap (type I)

Morphology

Ten completely filled neurons showed significant branching and terminations in the base, subcap and cap regions of OL glomeruli. All of these neurons had small (8–10 μm diameter)

Table 1. *Extent of glomerular innervation and response latencies of olfactory lobe local interneurons*

	Type I	Type II	Type III	Type IV
Region innervated	All regions	Cap	Subcap	Base
Number of glomeruli innervated (estimated)	6–40	200–900	100–200	200–400
N	10	7	6	3
Supra. latency (ms)	20±2	19±7	22±3	17, 17
Threshold latency (ms)	87±52	31±12	30±2	17, 17
N	13	9*	3	2

Supra. latency, latency to first action potential at the highest intensities tested. Threshold latency, latency at threshold intensities.

*The two type II interneurons showing a different response pattern were not included (see text for explanation).

Values are means ± S.E.M. unless *N*=2, when both values are given.

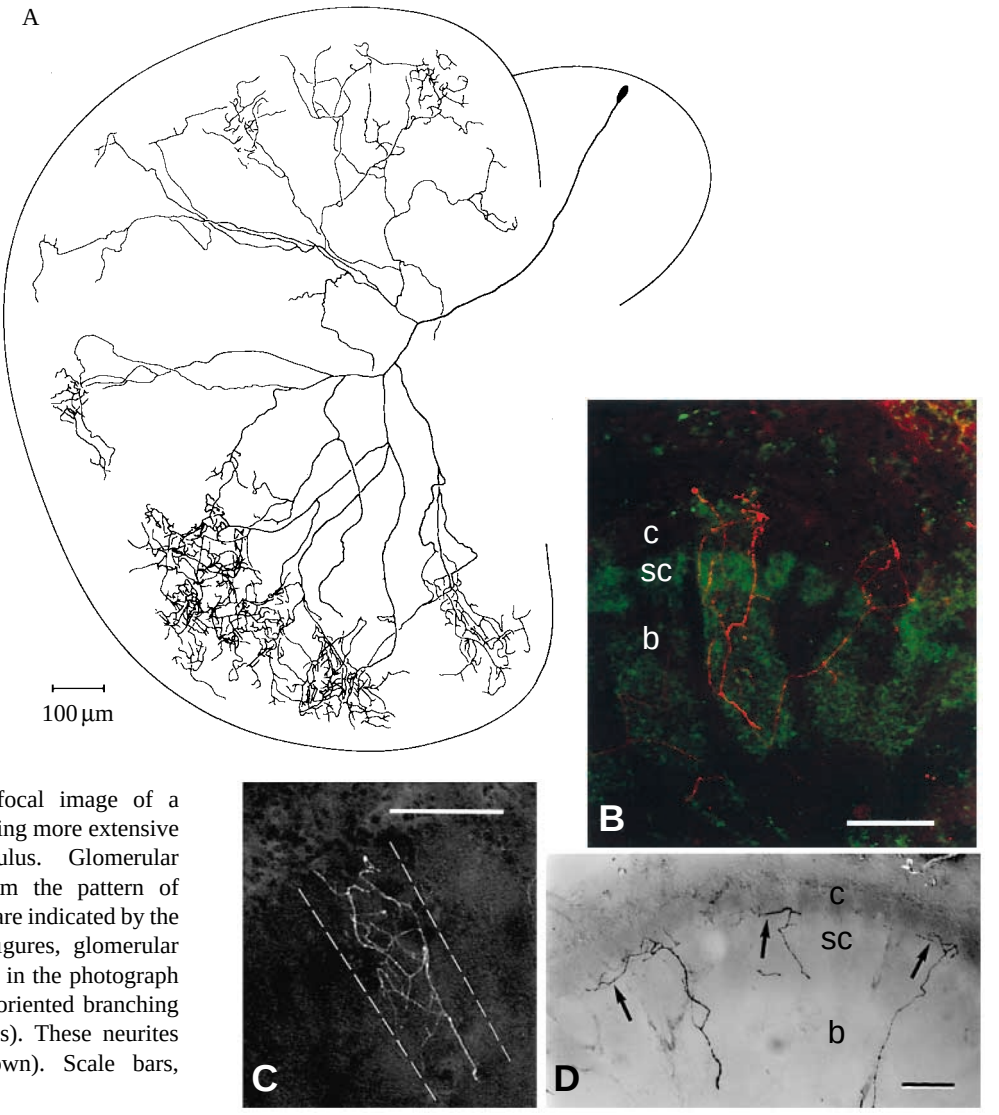

Fig. 2. Morphology of type I interneurons. (A) Serial reconstruction of a type I interneuron. (B) Confocal image of a type I interneuron (red) innervating two olfactory lobe (OL) glomeruli (green). Glomeruli are labeled with an antibody to histamine, allowing the cap (c), subcap (sc) and base (b) to be easily distinguished (see text for further explanation). Branches terminate in all three glomerular regions, and terminal branches do not cross glomerular boundaries. (C) Confocal image of a different branch of the neuron in A, showing more extensive branching within a single glomerulus. Glomerular boundaries and regions, visualized from the pattern of histamine antibody labeling (not shown), are indicated by the dashed lines. In this and subsequent figures, glomerular borders visible in the preparation but not in the photograph are indicated in this way. (D) Laterally oriented branching of neurites in the subcap region (arrows). These neurites cross glomerular boundaries (not shown). Scale bars, 100 μm.

somata located near the ventral surface of the medial cluster and neurites that entered the core of the OL and innervated a number of widespread glomeruli. Five additional neurons were less well filled, but could still be seen to innervate all three glomerular regions.

All of the 10 completely filled neurons innervated a relatively small number of OL glomeruli: most innervated 20–40 glomeruli, but one innervated only six glomeruli (Table 1). Three of the neurons, including the one reconstructed in Fig. 2A, were asymmetric in their branching, innervating more glomeruli in the anterior or posterior regions of the OL. Generally, one or two neurites entered a glomerulus proximally, then gave rise to 5–10 branches terminating in all glomerular regions (Fig. 2B). Neurites occasionally branched more densely, with 20 or more terminal branches per glomerulus (Fig. 2C). The branches of type I interneurons generally remained confined to a particular glomerulus (Fig. 2B,C). While this restricted branching was the most prevalent pattern seen in the type I interneurons, each neuron also had several branches that passed through the base of a glomerulus and then spread laterally in the subcap, innervating 2–4 adjacent glomeruli (Fig. 2D). Thus, neurites that branched across glomerular boundaries nearly always did so in the subcap region.

Physiology

Thirteen neurons of this morphological type were characterized according to their response to electrical stimulation of the antennular nerve. All showed a similar response pattern consisting of an initial burst of action potentials followed by a prolonged depolarization (Fig. 3). The duration of the spike burst and depolarization was graded with stimulus intensity, ranging from a single spike and a 1 s depolarization at near-threshold intensities, to up to 20 spikes and a 10 s depolarization at maximal intensities. The latency to firing varied dramatically with stimulus intensity, ranging from a minimum of 15 ms at maximal intensities to a maximum of 178 ms at near-threshold intensities. The mean latencies at these intensities are reported in Table 1. At near-threshold intensities, spiking was always preceded by a slow-onset

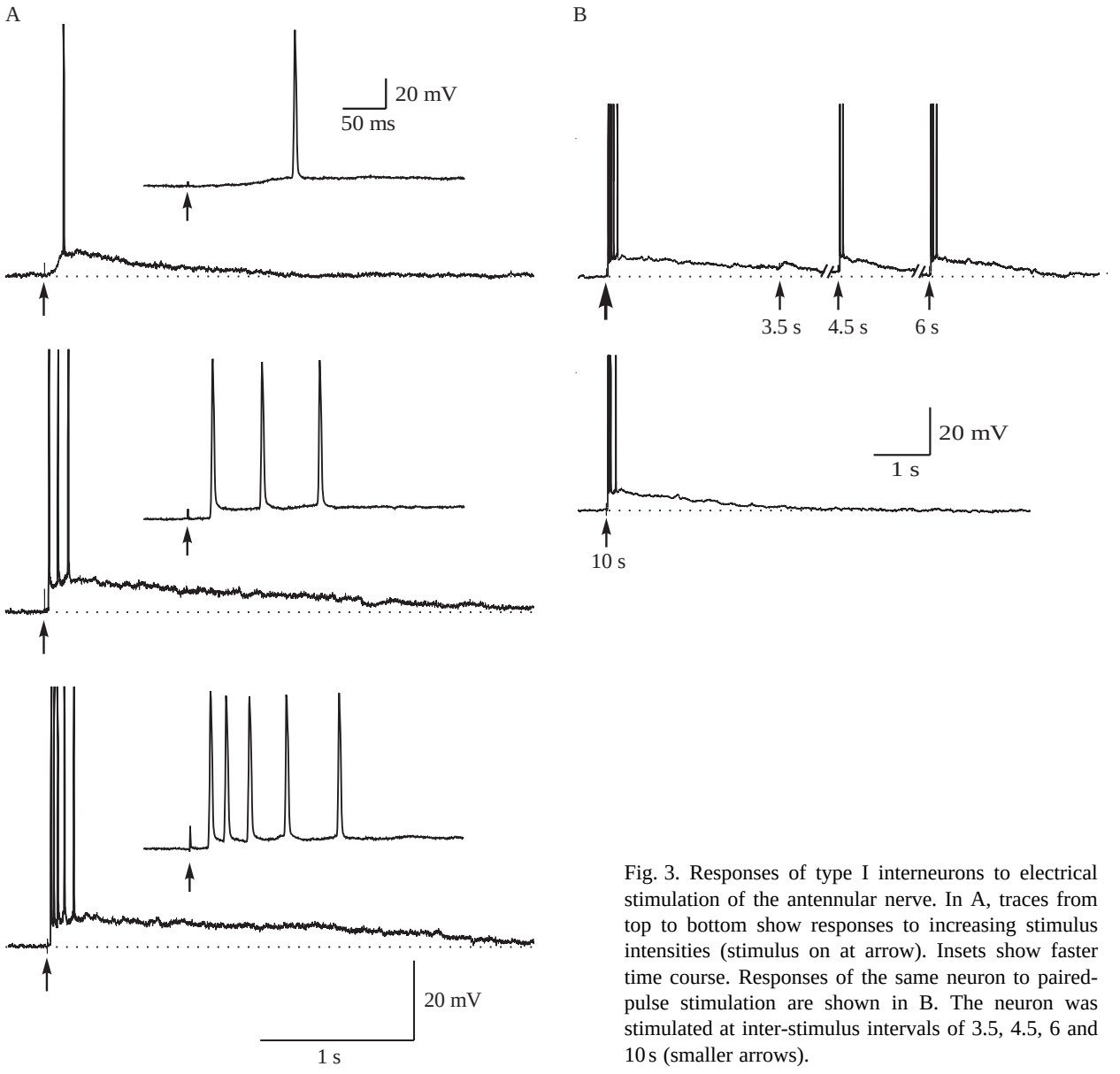

Fig. 3. Responses of type I interneurons to electrical stimulation of the antennular nerve. In A, traces from top to bottom show responses to increasing stimulus intensities (stimulus on at arrow). Insets show faster time course. Responses of the same neuron to paired-pulse stimulation are shown in B. The neuron was stimulated at inter-stimulus intervals of 3.5, 4.5, 6 and 10 s (smaller arrows).

depolarization that began 20–30 ms after stimulation (Fig. 3A). Subthreshold stimulus intensities elicited a slight (<10 mV) depolarization lasting 1–4 s.

As previously reported for OL projection neurons (Wachowiak and Ache, 1994), OL interneurons showed a prolonged period of suppression following a single conditioning stimulus. Responses to paired-pulse stimulation were tested in 11 type I interneurons. In nine cells, spiking responses were suppressed for 4–5 s after a conditioning stimulus (Fig. 3B), and in the other two cells suppression lasted 8 s. However, full recovery of the initial response pattern, as measured by response latency, spike burst duration and depolarization duration, took up to 10 s, indicating that a partial suppression persists for at least this long following a single nerve shock. In these and the other cells described (below), the duration of this suppression did not vary with stimulus intensity.

Neurons innervating the cap (type II)

Morphology

Eleven completely filled neurons branched exclusively in the cap region of many glomeruli (see Fig. 4). These neurons had larger (15–20 μm diameter) somata in the dorsal and lateral regions of the medial cluster and 3–4 major neurites around the periphery of the OL. Neurons of this morphological type appear to be identical to the ‘rim interneurons’ identified earlier in our laboraotry using microelectrode penetrations (Schmidt and Ache, 1996). Five additional neurons were less completely filled, but nonetheless branched exclusively in the cap region.

The number of glomeruli innervated by a single type II interneuron varied, ranging from as few as 15 % to as many as 85 % of the approximately 1100 OL glomeruli (Table 1). Five of the neurons branched asymmetrically, innervating many glomeruli in the ventral and anterior regions of the OL and few

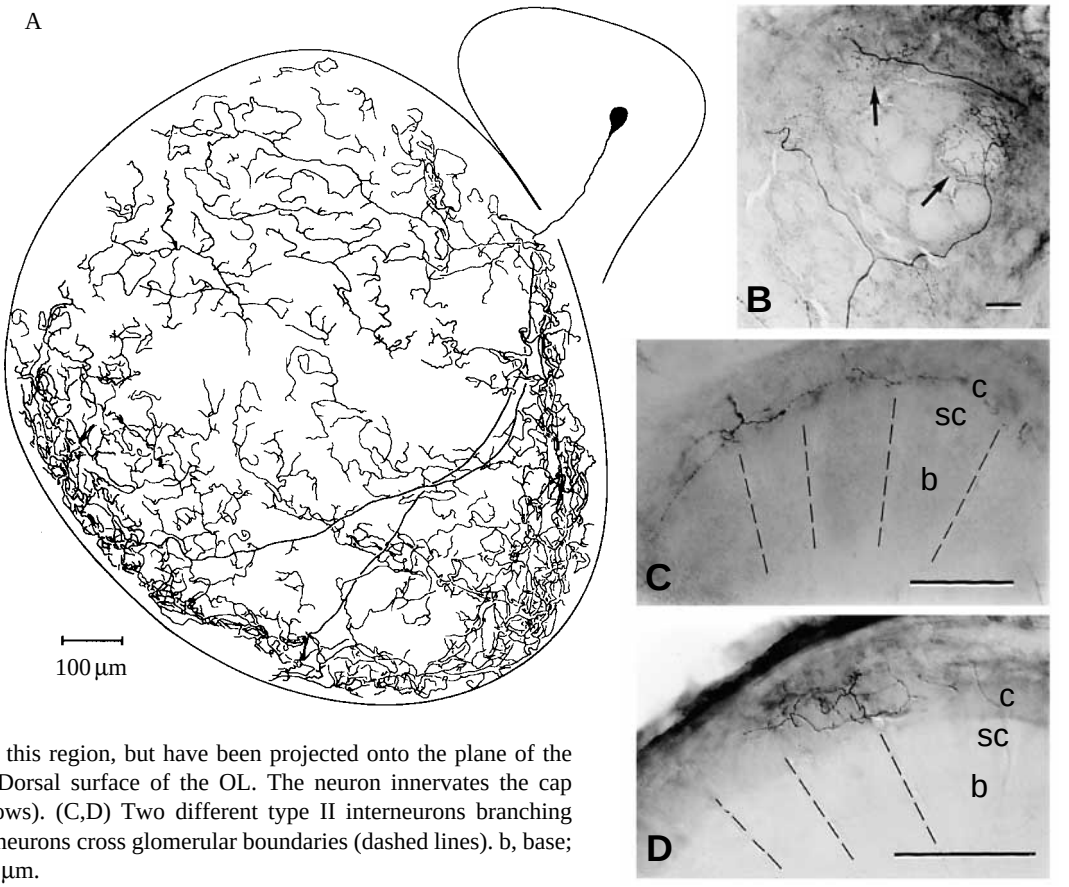

Fig. 4. Morphology of type II (cap) interneurons. (A) Serial reconstruction of a type II interneuron. The neuron travels acround the periphery of the olfactory lobe (OL) and branches in the cap region of many glomeruli. Processes appearing in the center of the OL actually innervate the caps of glomeruli in this region, but have been projected onto the plane of the paper in this reconstruction. (B) Dorsal surface of the OL. The neuron innervates the cap regions of several glomeruli (arrows). (C,D) Two different type II interneurons branching exclusively in the cap region. The neurons cross glomerular boundaries (dashed lines). b, base; sc, subcap; c, cap. Scale bars, 100 µm.

or no glomeruli in the more lateral regions (Fig. 4A). All of the neurons branched densely in the cap, typically giving rise to 5–10 terminal branches per glomerulus (Fig. 4B–D). These branches often crossed into adjacent glomeruli before terminating, but always remained confined to the cap region (Fig. 4C).

Physiology

Of thirteen type II interneurons characterized physiologically, 11 showed a similar pattern of response to electrical stimulation, consisting of an initial burst of 1–3 action potentials atop a 5–10 mV depolarization, followed by a slow-onset hyperpolarization lasting from 500 ms to 1 s (Fig. 5A). In one cell, the hyperpolarization lasted up to 8 s (Fig. 5B). In contrast to the action potentials seen in the other cell types, those of the type II interneurons appeared attenuated, with an amplitude of 20–40 mV. In all cells, the duration of the hyperpolarization, but not the duration of the initial spike burst, increased with increasing stimulus intensities (Fig. 5A). The response latency also varied with stimulus intensity, ranging from a minimum of 10 ms to a maximum of 43 ms (see Table 1 for mean latencies). Subthreshold stimulus intensities elicited a similar response pattern, but with no spike burst. Six of the cells were tested with paired-pulse stimulation. Suppression of the evoked spiking response lasted for 700 ms in one cell, 2–3 s in four cells and 6 s in the remaining cell. Full recovery of the initial spike latency and hyperpolarization generally took 2–3 s longer than the period of spike suppression.

Spontaneous, ongoing fluctuations in membrane potential, 1–3 mV in amplitude, were observed in nine of these 11 cells (Fig. 5A,B). While the fluctuations were somewhat irregular, they appeared to have a strong oscillatory component with a frequency of 1–2 Hz. Electrical stimulation inhibited the fluctuations for 1–2 s. In three of the cells, single spikes or brief bursts of 2–3 spikes sometimes occurred at the peak of the fluctuation. Hyperpolarizing the cell slightly eliminated action potential firing but did not affect the ongoing changes in membrane potential (Fig. 5B).

Two type II interneurons showed a different response pattern consisting of a single spike burst (Fig. 5C) whose duration was graded with stimulus intensity and ranged, in one cell, from 130 to 300 ms and, in the other, from 570 ms to 1.7 s. The response latencies were 15 and 19 ms, respectively, at maximal intensities and 29 and 60 ms at near-threshold intensities. Only in the first cell was the recording quality sufficient to detect subthreshold changes in membrane potential. In this cell, subthreshold intensities elicited only a small depolarization. This cell was also tested with paired-pulse stimulation and was suppressed for 3 s following a conditioning stimulus, although full recovery of the initial response took over 6 s. No spontaneous oscillations were observed in this cell. These two neurons were morphologically indistinguishable from the other type II interneurons.

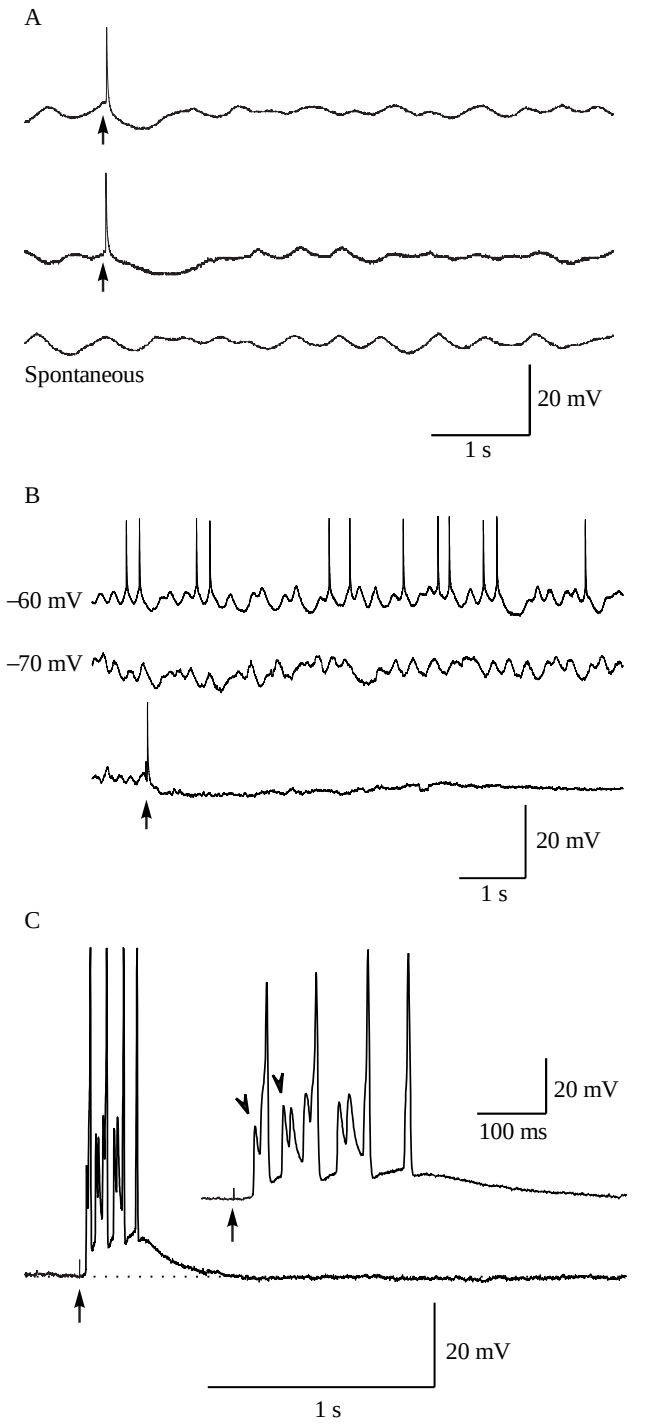

Fig. 5. Evoked and spontaneous activity of type II (cap) interneurons. Top two traces in A show responses to low- (top) and high- (middle) intensity stimulation. Bottom trace illustrates spontaneous fluctuations in membrane potential. In B, the top two traces show spontaneous activity at holding potentials of −60 mV and −70 mV. Note that no action potentials are seen at −70 mV, but the frequency of the fluctuations is unchanged. Bottom trace: response of the neuron to electrical stimulation. Note the prolonged hyperpolarization. Traces in C show the response pattern seen in two type II interneurons, consisting of a 100–200 ms burst of action potentials with no hyperpolarization. Note two different action potential amplitudes (arrowheads, inset).

Neurons innervating the subcap (type III)

Morphology

Six neurons with complete fills branched primarily or exclusively in the subcap region of OL glomeruli (see Fig. 6). All of the neurons had small (8–10 μm diameter) somata in the ventral region of the medial cluster, primary neurites which entered the core of the OL and smaller neurites which passed through the base region and then extended laterally in the subcap. Two additional, less well-filled neurons also showed extensive branching in the subcap.

Four of the six well-filled neurons had branches passing through the base regions of 20–40 glomeruli that, once reaching the subcap, gave rise to several laterally oriented branches innervating 2–5 adjacent glomeruli (Fig. 6). Thus, each neuron innervated approximately 100–200 glomeruli (Table 1). When the neurons were reconstructed across serial sections, nearly all neurites could be seen to terminate in the subcap (Fig. 6A,B), with few if any terminations in the base or cap regions. Most of the neurites branched sparsely, with typically less than five terminal branches per glomerulus.

Two of the six neurons had neurites extending across the subcaps of many adjacent glomeruli and thus innervated approximately 75 % of all 1100 OL glomeruli. These neurites also branched more extensively, typically with 10 or more terminal branches per glomerulus. Despite this more extensive branching, the neurites remained confined to the subcap. One of these two neurons was characterized physiologically, and its response properties were similar to those of the other type III interneurons (see below).

Physiology

Three type III interneurons were characterized physiologically, with all three showing a similar pattern of response to electrical stimulation. This pattern consisted of a 50–200 ms burst of 1–6 action potentials, followed by a very small, delayed depolarization lasting from 500 ms to 4 s (Fig. 7A). While the duration of the depolarization increased with increasing stimulus intensity, the duration of the initial burst did not. The response latency varied only slightly with stimulus intensity, ranging from 19 to 33 ms (see Table 1 for mean latencies). Subthreshold intensities elicited a small depolarization lasting 200–300 ms, which, in two of the cells, was followed by a 1–2 mV hyperpolarization. Responses to paired-pulse stimulation were tested in two cells. In both, action potentials were suppressed for 6 s following a single conditioning pulse (Fig. 7B).

Neurons innervating the base (type IV)

Morphology

Three neurons, all of which were completely filled, branched primarily or exclusively in the base region of OL glomeruli. Each neuron had a 10–12 μm diameter soma in the medial cluster and a primary neurite that entered the core of the OL and innervated 20–35 % of the approximately 1100 OL glomeruli (Fig. 8; Table 1). The neurons branched densely in

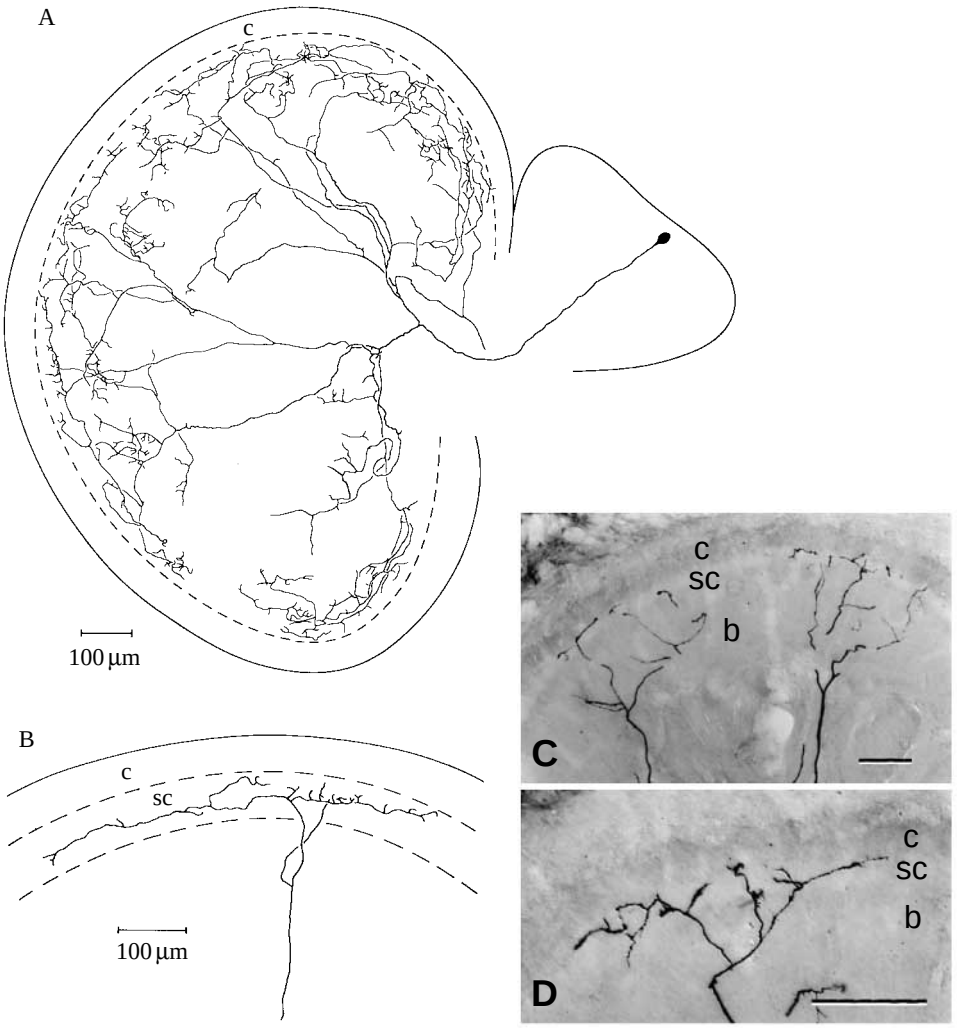

Fig. 6. Morphology of type III (subcap) interneurons. (A) Serial reconstruction of a type III interneuron. Dashed line indicates approximate border between cap and subcap. Terminal branches appearing proximal to the subcap are due to the projection of the reconstruction onto the plane of the paper. (B) Detail of reconstruction in A, showing a single neurite giving rise to laterally oriented branches in the subcap region. (C–D) Details of glomerular innervation by a different type III interneuron. Branches terminate only in the subcap. b, base; sc, subcap; c, cap. Scale bars, 100 µm.

the base region, typically giving rise to 15–20 branches per glomerulus. Only a very few branches could be seen to extend beyond the base region before terminating (Fig. 8A). While neurites often crossed glomerular boundaries, the majority of the branches were oriented parallel to the long axis of the glomerulus, and thus any one branch did not innervate many adjacent glomeruli (Fig. 8C,D).

Physiology

Of the three filled cells, two were characterized physiologically. Both neurons showed similar responses to low-intensity electrical stimulation, consisting of a 2–6 mV depolarization lasting approximately 30 ms followed by slightly larger hyperpolarization lasting approximately 1 s (Fig. 9A). The amplitudes of the depolarization and hyperpolarization increased with increasing stimulus intensity. In one of the neurons, the depolarization gave rise to a burst of 1–3 action potentials at higher stimulus intensities, always with a latency of 17 ms (Fig. 9B). This neuron was also tested with paired-pulse stimulation. The response to a second stimulus was suppressed for 6 s, with full recovery of the initial response pattern taking 10 s.

Discussion

We have characterized four distinct classes of local OL interneurons that are presumably involved in intrinsic processing of olfactory information (Fig. 10). In addition to the regionalized branching of three of the four classes, each of the four neuron types showed a different pattern of response to electrical stimulation of olfactory afferents in the antennular nerve. These findings suggest that the cap, subcap and base regions represent sites of functionally distinct steps in intrinsic processing within the OL.

As the medial cluster contains the somata of approximately 91 000 neurons (Schmidt and Ache, 1996), there may be additional types of local OL interneurons that we failed to detect. However, between this and a previous study of other deutocerebral interneurons with medial cluster somata (Wachowiak *et al.* 1996), we have now sampled approximately 70 neurons in widespread regions of the cluster and have so far failed to detect additional types of local OL interneurons. Thus, we tentatively conclude that the four classes described in this study represent the principal, if not the only, neuron types intrinsic to the OL.

All of the interneurons were multiglomerular and branched

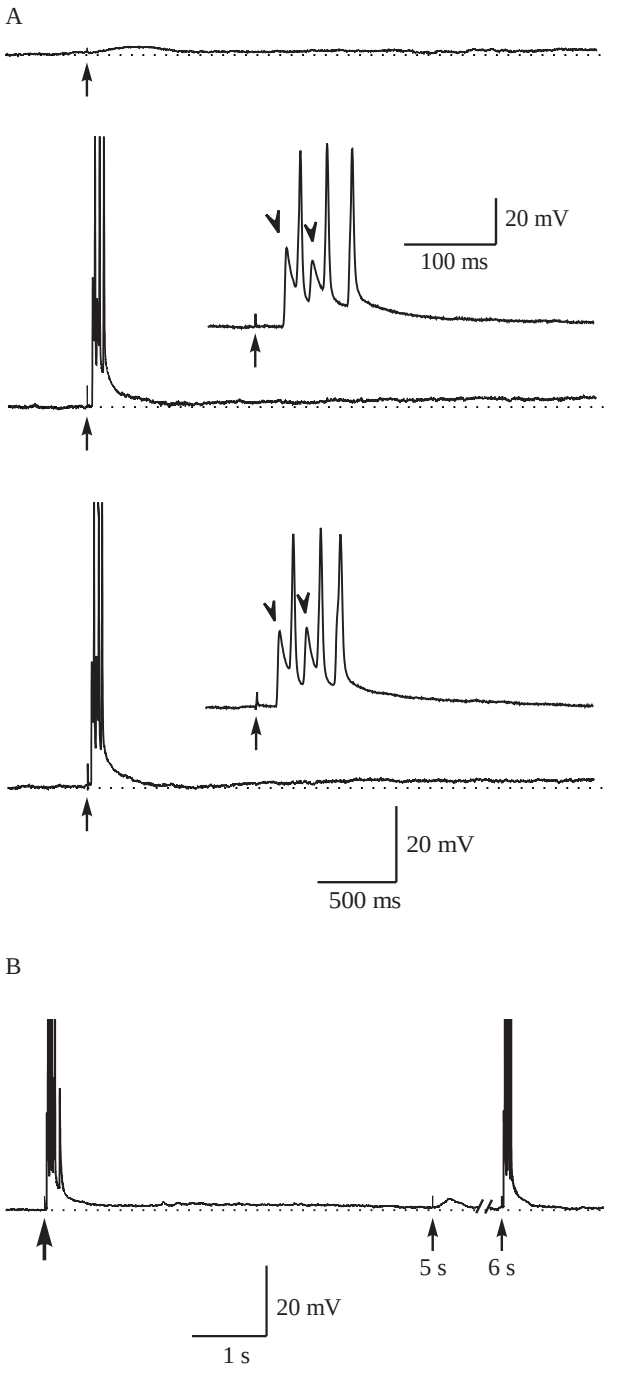

Fig. 7. Responses of a type III (subcap) interneuron to electrical stimulation. In A, traces from top to bottom show responses to increasing stimulus intensities. A subthreshold intensity stimulus (top) elicits a small depolarization. Note multiple action potential amplitudes (arrowheads, insets). (B) Response of the same neuron to paired-pulse stimulation, tested at intervals of 5 and 6 s (small arrows).

widely across the OL. None of the neurons had identifiable axons, even though they supported action potentials. The multiglomerular, anaxonal nature of these neurons is consistent with features of local olfactory interneurons in other arthropods, which innervate many or all glomeruli and are anaxonal (Matsumoto and Hildebrand, 1981; Ernst and Boeckh, 1983; Fonta *et al.* 1993; Mellon and Alones, 1994). Some of the cells we describe are consistent with earlier evidence for regionalized branching within olfactory glomeruli of crustaceans. The type II interneurons identified in this study appear identical to the 'rim interneurons' characterized by Schmidt and Ache (1996) using microelectrode penetrations. Mellon and Alones (1994) have identified a class of intrinsic interneurons that terminates primarily in the subcap region of OL glomeruli in crayfish *Procambarus clarkii*. Extrinsic neurons that connect the OL to other deutocerebral neuropils also show regionalized branching within lobster glomeruli (Schmidt and Ache, 1996; Wachowiak *et al.* 1996). Thus, the subdivision of glomeruli into cap, subcap and base regions appears to be a major principle underlying the organization of glomeruli in the crustacean OL, affecting many, if not all, aspects of glomerular processing.

Functional properties of intrinsic OL interneurons

The finding that three of the interneuron classes selectively innervate the three glomerular regions indicates that all three regions play distinct roles in the intrinsic processing of olfactory information in the OL. It is difficult to infer a great deal about the function of the cap, subcap and base without more detailed information about the synaptic organization of these regions. For example, receptor cells could potentially provide direct input to each of the four interneuron types identified here (as well as to projection neurons) since receptor cell axons extend throughout the length of the glomerulus and terminate in the cap, subcap and base regions (Schmidt and Ache, 1992). However, the heterogeneous branching patterns of the local OL interneurons, as well as those of projection neurons, provide important constraints on the possible synaptic connections that can occur in the different regions.

Several lines of evidence suggest that neurons innervating all three regions (type I) and those selectively innervating the cap region (type II) mediate inhibitory synaptic interactions in OL glomeruli. We have shown previously that projection neurons, the primary output neurons of the OL, respond to electrical stimulation with a brief excitation followed by a long-lasting hyperpolarization (Wachowiak and Ache, 1994). The hyperpolarizing phase can be blocked by antagonists to lobster histamine receptors and enhanced by γ-aminobutyric acid (GABA) receptor antagonists (Wachowiak and Ache, 1997). The type I interneurons respond to electrical stimulation with a depolarization that closely matches the time course of the hyperpolarization in projection neurons and recover from a conditioning pulse with a time course similar to the recovery of the projection neuron hyperpolarization. The type I interneurons also have morphological properties that match the pattern of histamine-like immunoreactivity in OL glomeruli in that they innervate all three glomerular regions, but branch most extensively in the subcap, while histamine-like immunoreactivity is found in all three glomerular regions but is greatest in the subcap (Wachowiak and Ache, 1997). Finally, preliminary studies have shown that GABA receptor

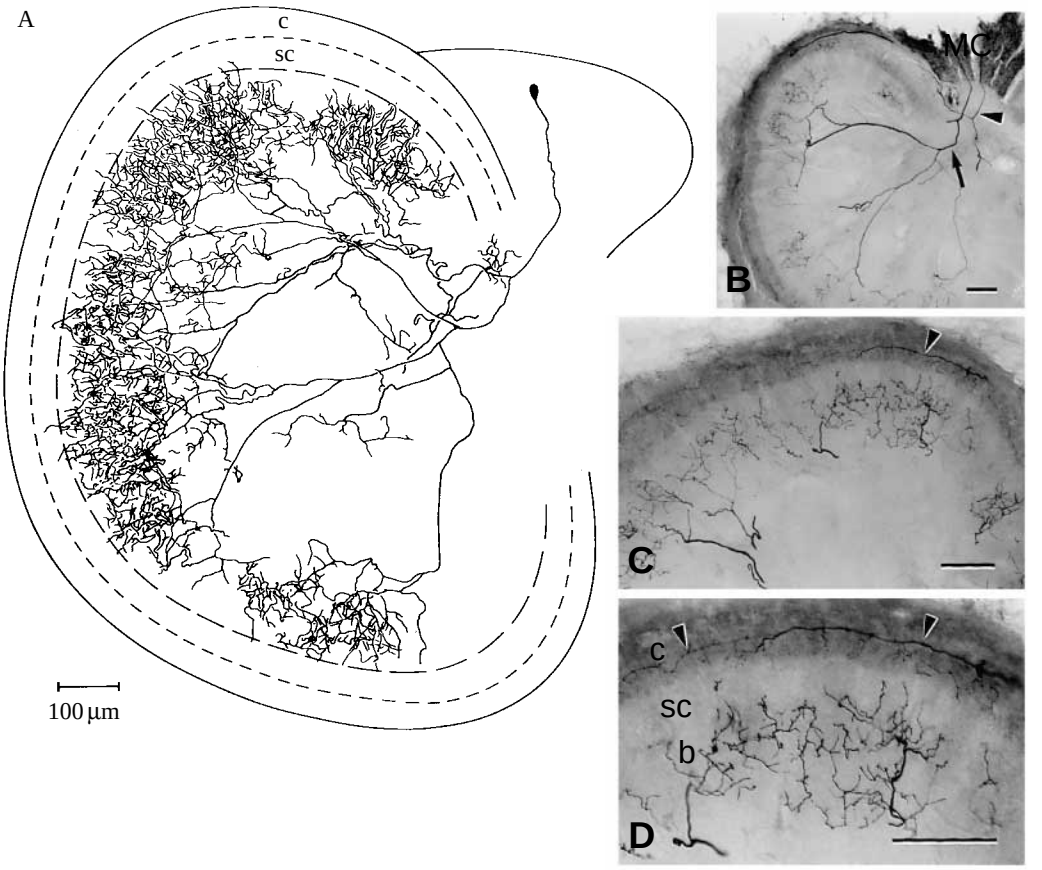

Fig. 8. Morphology of type IV (base) interneurons. (A) Serial reconstruction. Dashed lines indicate approximate borders between cap, subcap and base. (B–D) Details of glomerular innervation by neuron in A. A type II interneuron is also filled (arrowheads). Arrow in B indicates the primary neurite of the type IV interneuron. MC, medial cluster; b, base; sc, subcap; c, cap. Scale bars, 100 µm.

antagonists increase the stimulus-evoked depolarization of the type I interneurons (M. Wachowiak, unpublished observations), an effect which parallels the increased hyperpolarization of projection neurons following GABA receptor blockade. Thus, we propose that the type I interneurons are histaminergic and mediate the hyperpolarization of OL projection neurons following electrical stimulation.

The type II (cap) interneurons have morphological properties that closely match the pattern of GABA-like immunoreactivity in the OL. GABA-like immunoreactivity is found in the cap regions of all glomeruli, as well as in fiber tracts containing the primary neurites of the type II interneurons, and is highest in the same region of the medial cluster as that containing the type II interneuron somata (Wachowiak and Ache, 1997). Thus, we hypothesize that the type II interneurons are GABAergic. Previous work has shown that GABA acts as an inhibitory transmitter in the OL and can regulate the sensitivity of projection neurons to olfactory input (Wachowiak and Ache, 1997).

However, since projection neurons branch primarily in the base and subcap, only rarely innervating the cap (Wachowiak and Ache, 1994), the type II interneurons in the cap probably do not directly shape projection neuron responses. Thus, intrinsic processing in the cap occurs presynaptic to the projection neurons themselves. The fact that, in preliminary experiments, GABA receptor antagonists increase the stimulus-evoked depolarization of type I interneurons (see above) suggests that one role of the type II interneurons is to provide inhibitory input to the type I interneurons. However, since the same antagonists also increase the sensitivity of projection neurons to olfactory input (Wachowiak and Ache, 1997), the type II interneurons may have an additional role in modulating the transfer of excitatory input to projection neurons indirectly by presynaptically inhibiting receptor cell axon terminals. While we currently have no direct evidence for presynaptic afferent inhibition by type II interneurons, this possibility is made more likely by the fact that type I interneurons and receptor cell axon terminals are currently the only other neurons known to innervate the cap.

The subcap (type III) and base (type IV) interneurons were less well characterized in this study, so their respective roles in shaping OL output are less clear. The electrically evoked responses of type III and type IV interneurons were more similar to each other than to those of any other neuron types. However, the response patterns were clearly distinct, since type III interneurons did not show a hyperpolarization following the initial action potential burst, while both of the physiologically characterized type IV interneurons did hyperpolarize. Since histamine-like immunoreactivity is highest in the subcap (Wachowiak and Ache, 1997), the subcap interneurons may, in addition to the type I interneurons, mediate histaminergic inhibition in the OL.

All of the neuron types showed prolonged suppression of

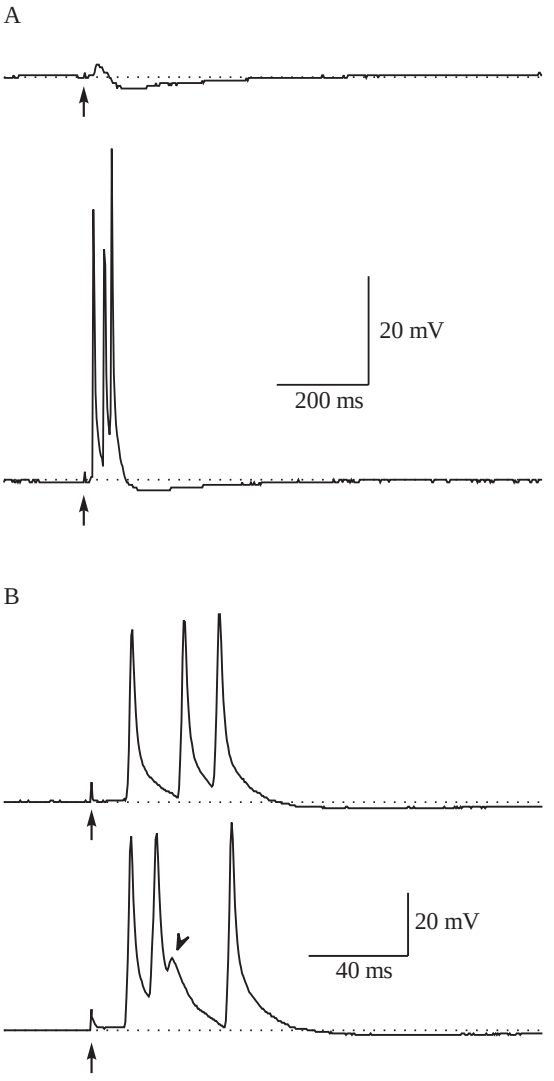

Fig. 9. Responses of a type IV (base) interneuron to electrical stimulation. In A, the top trace shows the response to a subthreshold intensity stimulus. The lower trace shows the response to a stimulus of suprathreshold intensity. Traces in B show the responses of the same neuron to near-threshold (top trace) and maximal (lower trace) intensity stimuli. Note the smaller-amplitude action potential in the lower trace (arrowhead).

evoked responses following a single shock to the antennular nerve, generally lasting 3–10 s. OL projection neurons also show paired-pulse suppression, although of an even longer duration (Wachowiak and Ache, 1994). As with suppression in projection neurons, the mechanism underlying the prolonged suppression of local interneuron responses is unknown. The suppressive effect may be much less intense when receptor cells are activated by odors and may be important in shaping the responses of OL neurons during repetitive odor stimulation.

Given the extent of the dendritic arborization of the interneurons, all of which lack an axon, it seems likely that restricted dendritic regions might participate in local processing of synaptic events in a relatively independent manner, as has been proposed for granule cell interneurons in

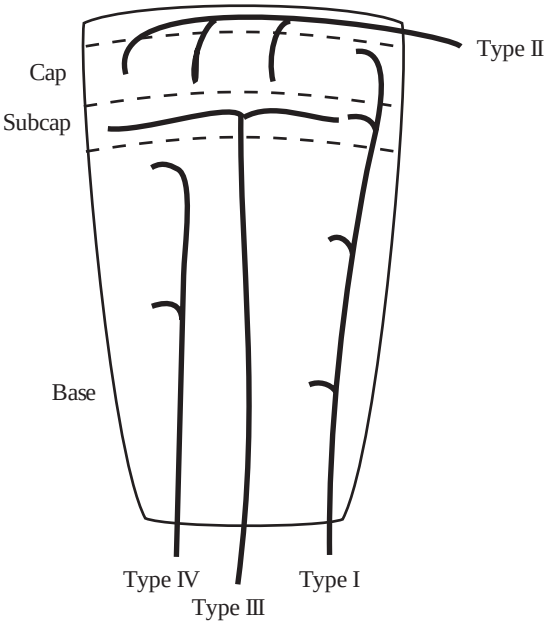

Fig. 10. Schematic illustration of the branching patterns of types I–IV local interneurons.

the mammalian olfactory bulb (Woolf *et al.* 1991). In support of this idea, we often observed action potentials of multiple height in OL interneurons (see Figs 5, 7 and 9), a feature which has also been noted for olfactory interneurons in the crayfish *Procambarus clarkii* and in the moth *Manduca sexta* (Mellon and Alones, 1994; Matsumoto and Hildebrand, 1981). The extent to which different regions of the same neuron function independently is an unknown factor that is of critical importance to how these neurons process information between and within glomeruli.

Importance of intraglomerular heterogeneity

Recent studies in insects have demonstrated that glomeruli can be heterogeneous with respect to their synaptic organization or the branching patterns of olfactory neurons. Glomeruli in the honeybee antennal lobe have two distinct tissue layers, with some local interneurons branching only in the inner layer (Fonta *et al.* 1993) and the highest density of synapses located in the outer layer (Gascuel and Masson, 1991). In the moth *Manduca sexta*, receptor cell axons branch primarily in the outer glomerular regions while projection neurons and other extrinsic interneurons branch primarily in the inner regions (Sun *et al.* 1993; Homberg *et al.* 1995). In the cockroach *Periplaneta americana*, input synapses to projection neurons predominate in the outer part of the glomerulus while output synapses are found in the inner regions (Malun, 1991).

In mammals, receptor cell axon terminals and the dendrites of projection neurons (mitral/tufted cells) and local interneurons (periglomerular cells) can branch in restricted regions within a single glomerulus (Hálasz and Greer, 1993; Takami and Graziadei, 1991; Shipley and Ennis, 1996). Evidence also

suggests that, as in insects, mammalian glomeruli may consist of concentric layers which are preferentially innervated by different neuron types (Greer *et al.* 1995). Thus, functional heterogeneity and compartmentalization within olfactory glomeruli may be a general feature of the first synaptic level of the olfactory pathway. This organization might enable more effective coding of odor inputs by second-order neurons, as shown in a modeling study comparing odor processing by glomeruli consisting of either one or multiple subcompartments (Schild and Riedel, 1992).

The nature of glomerular heterogeneity may differ somewhat between vertebrates and arthropods. While vertebrate glomeruli appear to contain subcompartments defined by the restricted branching of receptor cells or second-order neurons, little evidence exists for an ordered subdivision of glomeruli into functionally distinct regions, as is shown here for a crustacean and is probably the case for other arthropods. The vertebrate olfactory bulb, however, is divided into functionally distinct layers (glomerular and plexiform layers), and one must consider the possibility that the subcompartments in arthropod glomeruli are in part analogous to these layers, since arthropod glomeruli encompass all synaptic processing events at this level (Tolbert and Hildebrand, 1981; Sandeman and Luff, 1973), while in vertebrates processing occurs both in glomeruli and in the plexiform layers (Shipley and Ennis, 1996).

In the sense that a defining feature of olfactory glomeruli is the presence of contacts between receptor cell axons and second-order neurons, however, each of the three glomerular regions in the lobster probably has some functional analogy to vertebrate glomeruli, since receptor cell axons terminate throughout the length of the glomerulus (Schmidt and Ache, 1992). Given the diversity of neuron types identified in the crustacean OL and the complexity of their response patterns (Schmidt and Ache, 1996; Mellon and Alones, 1994), it is almost certain that the synaptic interactions between receptor cells and second-order neurons are as complex as those described for vertebrate glomeruli (Pinching and Powell, 1971; White, 1972). The present study suggests that, in the lobster OL glomerulus, receptor cells contact different neuron types in different glomerular regions. Thus, as more is learned about the synaptic interactions between the various neuron types in the OL, the columnar, layered organization of the OL glomerulus should aid in understanding the role of these multiple pathways in olfactory processing at the first synaptic level.

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